B-lymphoblastic lymphomas evolving from follicular lymphomas co-express surrogate light chains and mutated gamma heavy chains

Running title: B-lymphoblastic lymphomas originating from follicular lymphomas

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Abstract

Follicular lymphoma (FL) is an indolent B-cell non-Hodgkin lymphoma able to transform into germinal center-type diffuse large B-cell lymphoma (DLBCL). We describe four extraordinary cases of FL, which progressed to $TdT^{+}CD20^{-}$ precursor B-lymphoblastic lymphoma (B-LBL). FISH analysis showed that all four B-LBLs had acquired a *MYC* translocation upon transformation. Comparative genomic hybridization analysis of one case demonstrated that in addition to 26 numerical aberrations that were shared between the FL and B-LBL, deletion of *CDKN2A/B* and 17q11, 14q32 amplification and copy-neutral LOH of 9p were gained in the B-LBL cells. Whole-exome sequencing revealed mutations in *FMN2*, *NEB* and *SYNE1* and a nonsense mutation in *KMT2D*, all shared by the FL and B-LBL and *TNFRSF14*, *SMARCA2, CCND3*mutations uniquely present in the B-LBL. Remarkably, all four FL – B-LBL pairs expressed IgG. In two B-LBLs, evidence was obtained for ongoing rearrangement of immunoglobulin (*IG*) light variable genes and expression of the surrogate light chain. *IGHV* mutation analysis showed that all FL – B-LBL pairs harbored identical or near-identical somatic mutations. Based on the somatic gene alterations found in the *IG* and non-*IG* genes, we conclude that the FLs and B-LBLs did not develop in parallel from early t(14;18)-positive *IG* unmutated precursors, but that the B-LBLs developed from preexistent FL subclones that accumulated additional genetic damage.

Introduction

Follicular lymphomas (FLs) are the prototypic germinal center (GC)-derived B-cell non-Hodgkin's lymphomas [1] and cytologically and histologically resemble normal GCs. In accordance, FLs share a gene expression profile with normal centroblasts and centrocytes [2] and express somatically hypermutated B-cell antigen receptors (BCR), which in ~25% of cases are of the non-IgM isotype.[3, 4]

FLs have an indolent clinical behavior with a reported median survival of 8 to 10 years after diagnosis.[1] However, 20-60% of FLs transform into higher-grade malignancies, an event associated with an aggressive clinical course, poor therapy responses and short survival.[5] Usually, FLs transform into other GC-type lymphomas, most often diffuse large B-cell lymphomas (DLBCL) or, less commonly, Burkitt-like lymphomas (referred to in the latest WHO classification as B-cell lymphoma unclassifiable with features intermediate between DLBCL and Burkitt-lymphoma).[5, 6] Infrequently, these prototypic GC-like malignancies transform into precursor B-lymphoblastic lymphomas (B-LBL).[7-15] Studies on sporadic cases described so far revealed that such B-LBLs carried *BCL2* translocations, indicative for a clonal relationship with the preceding FLs. Karyotype analyses showed a variety of chromosomal alterations gained in the B-LBLs, among which *MYC* locus translocations were most frequently observed (in 18/19 (95%) of the described cases).[7-15] Lymphomas with two recurrent translocations, one of which being *MYC*, are referred to as "double-hit-B-cell lymphomas" and have a dismal prognosis.[6]

Here we analyzed four FLs that transformed into B-LBLs. We demonstrate that these B-LBLs phenotypically and functionally display key features of early B cells and that, in addition to *MYC* translocations, also *BCL6* gene translocation may accompany this infrequent mode of FL progression. Genome-wide copy number variation analysis and whole-exome sequencing (WES) of one of the transformed FLs demonstrated that a limited number of genomic alterations was gained in the B-LBLs, including deletions of the tumor suppressor genes *CDKN2A/B* and *NF-1* and mutations in *TNFRSF14*, *SMARCA2* and *CCND3*.

Materials and methods

Patient material.

The clinicopathological characteristics of the four patients with a FL that transformed into a B-LBL are shown in Table 1. This study was conducted in accordance with the ethical standards in our institutional medical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, revised in 1983.

Clinico-pathological history of the four patients.

TF 1: A FL grade I was diagnosed, which was treated with chlorambucil. After one year a B-LBL was diagnosed and the patient was treated with high dose chemotherapy consisting of iphosphamide, etoposide, methotrexate and two consolidation cycles with cytarabine, etoposide and methotrexate. Complete remission was reached. A few weeks later progression of the disease occurred and no further treatment was undertaken. The patient died subsequently. *TF 2*: A FL grade I was diagnosed, which was not treated. Two years later a B-LBL was diagnosed, which was treated with daunorubicin, vincristine, prednisone, asparaginase and methotrexate. Three months later the B-LBL reoccurred. The patient was treated with dexamethasone, but died a few weeks later. *TF 3*: A FL grade I was diagnosed. Two years later, laboratory evaluation showed a leukocyte count of 10.7 x 10^9/L with 38% atypical lymphocytes. Immunophenotyping demonstrated two lymphocytic populations, i.e. one population of mature monoclonal B cells and a distinct population of lymphoid blasts. Combined chemotherapy with cytarabin, etoposide and methotrexate was started. Complete remission was established following two additional courses of vincristine, dexamethason and adriamycine. Consolidation therapy consisted of cytarabin and asparaginase. Shortly thereafter the disease relapsed. Local radiotherapy was given. The patient died a few weeks later. *TF 4:* A FL was diagnosed, which was treated with 8 cycles of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP). A year later, PET-CT scan showed lesions in the abdominal wall and in the retroperitoneum, which pathologically turned out to be a DLBCL and a B-LBL, respectively. Rituximab combined with dexamethasone, high dose cytarabine and

cisplatin (R-DHAP) treatment was started but eventually the patient died several months later.

Immunohistochemistry.

Immunohistochemical stainings were performed on acetone-fixed cryostat sections and formalin-fixed paraffin-embedded (FFPE) tissue using Powervision^{+} detection system (Immunovision technologies, Daly City, CA). Monoclonal antibodies used: CD20 (B-Ly1), CD21-L (DRC-1), BCL2 (124), BCL6 (PG-B6P), CD79a (HM57), CD19 (HD37), Igλ (A193), Igκ (A191) (Dako, Glostrup, Denmark), CD10 (CALLA) and PAX5 (24) (Becton and Dickinson (BD), Erembodegem-Aalst, Belgium) and CD3 (SP7; Labvision, Neomarkers, Fremont, CA) were used. Terminal deoxynucleotidyl transferase (TdT) was stained with a polyclonal serum (Klinipath, Duiven, The Netherlands).

RNA isolation, cDNA synthesis

RNA was isolated using TRIZOL reagent (Invitrogen, Breda, the Netherlands) and complementary DNA (cDNA) was synthesized.[3] Immunoglobulin heavy chain variable region (*IGHV*) transcripts were amplified by PCR.[16, 17] In some experiments, *IGHV*-PCR products were cloned in pTOPO-TA-vectors and sequenced. *IGHV* genes were identified using V-Quest (http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=humanIg).[18] *VpreB* and *Lambda5* were amplified with the respective primer pairs: 5'-CTCATGCACTTTGTCTACTG-3' / 5'-CTGGCTCTTGTCTGATTGTGAG-3' and 5'-CTCCTGTCCTGCTCATGCTG-3' / 5'-GTACACACCGATGTCATGGTCG-3'.The *BCL2* PCR was performed on DNA, as described.[19]

Fluorescence in situ hybridization.

Chromosomal aberrations were investigated in 4 µm thick FFPE tissue sections by fluorescence in situ hybridization (FISH). A segregation-detection assay was used to detect breaks in *BCL2* (probe Y5407), *BCL6* (probe Y5408) and *MYC* (probe Y5410) (Dako).

Cell sorting

Lymph node suspensions of *TF3b* were stained with CD19-APC, CD10-PerCP-Cy5.5 and CD20-FITC monoclonal antibodies (BD). Cells were sorted in small $CD19^{+}CD10^{+}CD20^{lo}$ cells, small $CD19^{+}CD10^{+}CD20^{hi}$ cells, small $CD19^{+}CD10^{-}CD20^{hi}$ cells and large $CD19^{+}CD10^{+}CD20^{lo}$ cells by using a FACSAria (BD).

Comparative genomic hybridization-array

Genomic DNA was isolated using Qiagen's DNeasy kit and analyzed for copy number variation (CNV) using HumanCytoSNP-12 BeadChips (Illumina, San Diego, CA). Processing of DNA samples, hybridization, staining and scanning of the BeadChips were performed according to the Illumina Infinium II protocol at the array facility of ServiceXS (Leiden, the Netherlands). Data was analyzed using Illumina GenomeStudio software (version 2009.2). The LogR ratio (LRR) and the B allele frequency (BAF) data were processed into the OverUnder plugin as described previously[20] and copy numbers were calculated. Due to lack of normal tissue, it cannot be excluded that some of the CNVs are germline polymorphisms.

Whole-exome sequencing

Genomic DNA was amplified using the illustra GenomiPhi V2 DNA amplification kit (GE Healthcare, Little Chalfont, UK). Unamplified DNA of the B-LBL isolated from blood was used to control for amplification errors. Fragmentation was performed with the Covaris sonicator (GC Biotec, Alphen aan de Rijn, The Netherlands). Library preparation was performed using Solid chemistry. An exome capture was performed with Whole-exome capture EZ version 3 of Nimblegen (Roche, Almere, The Netherlands) with platform-specific adaptors and blockers. Clonal amplification of the captured library was performed by emulsion PCR (Life Technologies). Finally, samples were sequenced on a Solid 5500 xl sequencer. Obtained data was processed according to the NGS pipeline described by Houten et al.[21] Due to lack of normal tissue, only differential variants between FL and B-LBL could be determined. In addition, in our WES data we also checked for mutations in sets of 92 and 40 genes, previously found to be mutated in WES

data of FLs and B-cell acute lymphoblastic leukemias (B-ALLs), respectively (Supplementary Table 1).[5, 22-26] Eleven mutations, in 10 genes identified in *TF3b*, were validated by Sanger sequencing in *TF3b* and in addition, these 11 mutations were examined in *TF1a*, *TF2a* and *TF2c*. Moreover, EZH2, which is frequently mutated in FL, was screened for mutations in *TF1a* and *TF3b*.

Results

Histology and phenotype of four FLs before and after transformation into B-LBLs.

Before transformation, the four FLs (*TF1a-4a*) displayed a follicular growth pattern with a characteristic phenotype, i.e. $CD20^+$, $CD79a^+$, $BCL2^+$ cells co-expressing the GC markers CD10 and BCL6 that expanded in networks of follicular dendritic cells (Tables 1 and 2). After B-LBL transformation, the follicular architecture of *TF1*, *2* and *4* was lost. The tumor cells of all four cases still expressed CD79a, CD10 and BCL2, but were devoid of CD20. In addition, all B-LBLs expressed terminal deoxynucleotidyl transferase (TdT). In the *TF2b* B-LBL approximately 10% of the tumor cells stained positive for TdT. However, no TdT expression was observed in the *TF2c* biopsy. In *TF3b*, the follicular growth pattern was in part retained and alternated with sheets of lymphoblastic cells (Figure 1). To analyze the molecular features of the entities in *TF3b*, $CD19^+$ cells of lymph node suspensions were sorted by FACS based on size and expression of CD20 and CD10, in: (I) small $CD19^+CD10^+CD20^{lo}$ cells, (II) small $CD19^+CD10^+CD20^{hi}$ cells, (III) small $CD19^+CD10^-CD20^{hi}$ cells and (IV) large $CD19^+CD10^+CD20^{lo}$ cells. Both $CD20^{lo}$ fractions expressed TdT, as determined by RT-PCR, demonstrating that these fractions represent the B-LBL (Supplementary Figure S1). Of note, the FL of *TF4* transformed into a DLBCL before transforming into B-LBL (Table 1).

The four FLs and successive B-LBLs express clonally related somatically mutated IG gamma heavy chains.

IGHV gene analysis showed that in all patients the FL and B-LBL were of the same clonal origin, harboring identical *IGHV* rearrangements (Table 3). In *TF3b*, all four FACS-sorted fractions, including the small $CD19^+CD10^-CD20^{hi}$ cells, harbored an identical *IGHV* rearrangement, suggesting that this population is either a $CD10^-$ subclone of the FL or represents a stage intermediate between the FL and B-LBL. In accordance with their GC phenotype, all four FLs harbored somatically mutated *IGHV*. Intriguingly, all four lymphomas expressed IgG, as determined by RT-PCR, instead of the commonly found IgM in FL[4] (Table 3). Due to poor DNA quality, the *IGHV* of the *TF3a* FL could

only be investigated from framework 2 (FR2). We were unable to determine the *IGHV* of the *TF4b* DLBCL due to lack of material.

All four FL/B-LBL pairs harbored high numbers of shared somatic *IGHV* mutations (Figure 2), further underscoring their clonal relationship. Comparison of individual molecular *IGHV* clones of *TF1* and *TF3* showed intraclonal *IGHV* sequence variation (ICV) of the FLs but not of the B-LBLs. Particularly the $CD20^{hi}$ FL cell fractions of *TF3b* showed high ICV, whereas ICV was not detected in the $CD20^{lo}$ B-LBL cell fractions (Figure 2).

Some B-LBLs show ongoing light chain rearrangement and express surrogate light chains.

The FL *TF1a* was found to express an *IGLV2-18/IGLJ2/3a* rearrangement (Table 3). Interestingly, after transformation a different monoclonal immunoglobulin lambda variable region (*IGLV*) rearrangement was amplified out of the *TF1b* B-LBL, i.e. *IGLV3-21/IGLJ3b*. Amplification of *IGLV* of DNA followed by genescan analysis revealed that in both the FL and the B-LBL of *TF1* two *IGLV* rearrangements were present (data not shown). One of these *IGLV* rearrangements was of the same length before and after transformation. In accordance with the *IGLV* sequence analysis, the second *IGLV* rearrangement in the B-LBL of *TF1b* was of a different length than that of the FL of *TF1a*. The *IGLV3-21* gene segment, identified in the B-LBL, is located more upstream in the *IGLV* locus than the *IGLV2-18* gene segment of the FL. Most likely, the *IGLV3-21* rearrangement of the *TF1b* B-LBL has emerged by secondary rearrangement of the *IGLV*. Similarly, the *IGLV1-36/IGLJ3b* rearrangement detected in FL *TF2a* was not found in B-LBL *TF2b.* In *TF2b* an oligoclonal rearrangement pattern was seen by genescan analysis (data not shown). In all FACS-sorted cell populations of *TF3b*, an identical *IGLV4-69/IGLJ2/3a* rearrangement was observed. In accordance, a monoclonal *IGLV* region of identical length was observed by genescan analyses (data not shown).

The expression of the IG light chain was also assessed by IHC on cryostat sections. The tumor cells of both the FL and B-LBL of *TF1* expressed Igλ, but no Igκ (Table 2), confirming the RT-PCR analyses. In the B-LBL *TF2b*, Igλ was exclusively detected.

IGLV expression of FL *TF2a* and all *TF3* and *TF4* samples could not be investigated by IHC due to lack of frozen tissue.

It has been described that B-LBLs express the surrogate light chain (psi L), which is composed of two proteins, VpreB and λ5.[27] RT-PCR analyses revealed that both the B-LBL of *TF1b* and *TF2b* indeed expressed VpreB and lambda5, whereas the preceding FL of *TF1a* and *TF2a* did not (Figure 3). In *TF3a* and *TF3b*, VpreB and λ5 expression were not detected. Due to lack of material, VpreB and λ5 expression was not assessed in *TF4*.

All four B-LBLs acquired a *MYC* gene translocation.

In accordance with the immunohistochemical data, the FLs of *TF1a, TF3a* and *TF4a* harbored translocations of the *BCL2* gene as detected by segregation fluorescence in situ hybridization (FISH) and these translocations were also detected after histological transformation. Unfortunately, the *BCL2* FISH on *TF2a* was not interpretable, however, in the *TF2c* B-LBL a *BCL2* translocation was detected (Table 4). In all four patients, *MYC* gene translocations were identified after histological transformation. In addition, a 3q27 break, involving *BCL6*, was found in the FL of *TF1a,* whereas after progression no split signals were detected. In contrast, the B-LBL of *TF2c* showed a 3q27 break only after transformation. In *TF3* and *TF4,* a 3q27 breakage was not detected in any of the biopsies. Both in *TF3* and *TF4*, BCL6 protein expression decreased upon transformation, as detected by IHC (Figure 1).

Genome wide analyses of *TF3* before and after transformation.

To further uncover the genomic aberrations that accompanied progression of the FL of *TF3*, the sorted cell populations were analyzed by comparative genomic hybridization (CGH). The number of genomic aberrations in the FL of *TF3b* was similar to two control FLs. In total, the FL and B-LBL fractions of *TF3b* shared 26 genomic aberrations (Figure 4A), underscoring their clonal relationship. The shared aberrations comprised three amplifications, three deletions and copy-neutral loss of heterozygosity (CN-LOH) in 20 regions, including deletion of 22q11 and CN-LOH of 6q21 and 7q33, which are all commonly observed in FLs[28] (Table 5). Interestingly, these 26 shared aberrations also

contained regions previously described to be associated with transformation of FL into high-grade lymphomas other than B-LBL, such as deletion of 1p36.[28] It was noted by CGH that *MYC* was amplified in the FL prior to translocation (Figure 4A). The B-LBL harbored only a limited set of aberrations in addition to the aberrations already present in the FL, i.e. CN-LOH of 9p, amplification of 14q32 and deletion of 17q12. The affected region of 9p included CN-LOH of *JAK2* and *PAX5.* Importantly, a small region of 9p21 was deleted, which contains the tumor suppressor genes *CDKN2A/B* (encoding p15 and p16) and *MTAP* (Figure 4B). Genes amplified at 14q32 include the proto-oncogenes *TCL1*, *BCL11* and *AKT1 (PKB)*. The tumor suppressor gene *NF-1* is among the genes deleted on chromosome 17. In Supplementary Table S2, the affected genes in 9p, 14q32 and 17q12 are listed. In the $CD10^-$ fraction, an amplification of chromosome 12 was gained as compared to the FL fraction. It did however not share any of the aberrations found in the transformed B-LBL, suggesting that this fraction is a subclone of the FL rather than a tumor population at an intermediate stage between FL and B-LBL.

Whole-exome sequencing revealed heterozygous missense mutations in *CCND3*, *DYSF*, *ISOC2* and *ZFHX4* present in the B-LBL, which were absent in the FL. Two missense mutations (in *SMARCA2* and *SNAPC3*) heterozygously present in the FL, were hemizygous in the B-LBL due to LOH of chromosome 9p. In addition, one nonsense mutation in *TNFRSF14* and additional mutations in the 5'UTR of *BCL2* and *MYC* were found in the B-LBL (Table 6 and Supl. Figure 2). The mutations in *CCND3*, *ISOC2*, MYC and ZFHX4 were found in 7%, 9%, 3% and 6% of the sequence reads and thus were subclonal variants in the FL. Next to this analysis, we checked a set of genes previously described to be mutated in FL and B-ALL (see Materials and Methods). This unveiled missense mutations in *FMN2*, *NEB* and *SYNE1* as well as a nonsense mutation in *KMT2D*, shared by the FL and B-LBL. In addition, 1 synonymous, 2 3'UTR and 14 intronic mutations were identified (Supplementary Table 2). Eleven mutations in ten genes were validated by Sanger sequencing in *TF3b* (Supplementary Figure S2). These mutations were also examined in *TF1a*, *TF2a* and *TF2c* but were however not detected (data not shown). Moreover, no mutations in the commonly mutated gene *EZH2* were detected in *TF1a* and *TF3b*.The loss of *CDKN2A/B* as detected by CGH was confirmed

by comparing the numbers of sequence reads (data not shown). .

Discussion

Transformation of FL into a B-LBL occurs rarely and has been documented for a limited number of cases.[7-15] Here, we describe four B-LBLs that evolved out of FLs and show that, in spite of retained expression of mutated *IGHV* genes, the B-LBLs had acquired several key features of precursor B cells. The follicular architecture as well as CD20 expression were lost, the expression of *TdT*, *VpreB* and *Lambda5* was induced and evidence was obtained for reinitiated *IGLV* gene rearrangement since the expressed *IGLV* genes in the B-LBL of *TF1b* and *TF2b* were different from those of the FL of *TF1a* and *TF2a* respectively, while the *IGHV* genes were unaltered.

A t(14;18)(q32;q21) is a rare event in primary B-LBL/B-ALL. Stamatoullas *et al.* reported that of 142 adult B-ALLs, only five had a translocation involving the *BCL2* gene.[29] The Mitelman database contains 12 *de novo* B-LBLs that harbor both a *BCL2* and a *MYC* translocation.[30] In the studies assembled in the Mitelman database no *IGHV* sequence analyses were performed and thus it is not excluded that the $t(14;18)^+$ B-LBLs include cases that originate from FL.

Here, in all four lymphomas a *MYC* translocation was gained upon histological transformation. Secondary *MYC* translocations have been reported by others in 18 out of 19 FLs transforming into B-LBLs[7-15], suggesting that MYC overexpression is mandatory for transformation into a B-LBL. However, this event by itself is unlikely to impose an immature B-cell program given the fact that 10% of FLs transforming into a DLBCL also carry *MYC* rearrangements.[31] Remarkably, in the FL of *TF3, MYC* was amplified prior to translocation as determined by CGH. It has been reported in mice that *MYC* translocation is promoted by AID activity.[32] In this respect, the observations that the FLs of *TF2* and *TF3* display intraclonal *IGHV* sequence variation and that all four FLs express IgG, whereas the majority of FLs express IgM^2, are noteworthy and likely to be AID mediated. However, by RT-PCR we detected no AID expression in *TF1*, *TF2* or in *TF3* (data not shown). Interestingly, IgG expression was also noticed in two separately reported FLs that transformed into B-LBLs.[13, 14]

In *TF2,* progression to B-LBL was accompanied by a break at 3q27. Similarly, *BCL6* translocations have also been documented in other *de novo* t(14;18)$^{+}$ B-LBLs.[29] *BCL6* region translocations are known to occur in 6-10% of (grade 3) FLs[33], ~30% of DLBCL[33] and ~10% of FL transforming into DLBCL.[34] *TF2b* is a so-called "triple-hit lymphoma" carrying rearrangements of the *BCL2*, *BCL6* and *MYC* genes. This combination of genomic rearrangements has been reported before in sporadic cases of FL undergoing transformation to DLBCLs as well as in *de novo* DLBCLs.[6, 35, 36] Both the t(14;18)(q32;q21) and the t(8,14)(q22;q32) disrupt the *IG* coding region.[37, 38] Yet, the finding that in *TF2b IGHV* mRNA is still expressed, is not necessarily contradictory since *BCL6* locus rearrangements may engage the *IGL* chain or non-*IG* loci, while *MYC* may be juxtaposed to downstream *IG* switch regions thus leaving the *IG* coding region intact.[39] Alternatively, *BCL2* and *MYC* rearrangement may occur at the same *IGH* allele.[40] The phenomenon that in *TF1* the break at the 3q27 locus was no longer detected after progression into the B-LBL of *TF1b* has been reported in FLs transformed to DLBCL.[41]

High resolution CGH-array on sorted FL and B-LBL cells from *TF3b* revealed that the B-LBL had gained only a limited number of genetic aberrations, i.e. CN-LOH of 9p, deletion of 17q12, amplification of the oncogenes *TCL1*, *BCL11B* and *AKT*, all on chromosome 14 and amplification of 14q32 which includes the gene encoding the transcription factor Yin Yang 1 (*YY1*). *YY1* is expressed in early B cells and GC B cells, where it regulates *IGHV* -rearrangement and class switch recombination, respectively.[42] *YY1* has also been shown to activate the *MYC* promotor.[43] The deleted 9p region contains *CDKN2A/B* and *MTAP* and was previously described to be associated with transformation of FL into DLBCL.[5] Loss of *CDKN2A/B* also occurs in approximately 40% of adult B-ALLs and is associated with poor survival.[44] Deletion of the cell cycle regulators *CDKN2A/B* may be required for transformation since it renders the cells resistant to *MYC*-induced apoptosis.[5] It is, however, unlikely that *CDKN2A/B* loss is instrumental in the induction of a precursor B-cell phenotype.

PAX5, a master regulator of B-cell lineage commitment and differentiation[45] is among the genes on 9p that show loss of heterozygosity after transformation (listed in

Supplementary Table 2). Interestingly, mutations in *PAX5* were found in approximately 30% of *de novo* B-LBL, suggesting that loss of expression of this transcription factor results in a developmental blockade and drives B-LBL development.[45] Moreover, it was shown that *PAX5*$^{-/-}$ mice have a differentiation arrest at the pro-B-cell stage[45], whereas targeted deletion of *PAX5* in mature B cells resulted in loss of the B-cell phenotype and lymphoblast formation.[46] However, we did not detect mutations in the coding region of *PAX5* in *TF1*, *TF2* and *TF3* (data not shown) and PAX5 protein expression was measurable by IHC in all four B-LBLs (Table 2). Still, decreased PAX5 transcription and protein expression due to the 9p deletion cannot be excluded. Neither did we detect mutations in *IKZF1* in *TF3* (data not shown), another master regulator of B-cell commitment and differentiation, which is frequently deleted in *de novo* B-LBL.[45]

Whole-exome sequencing revealed missense mutations in *FMN2*, *NEB* and *SYNE1* as well as a nonsense mutation in *KMT2D*, shared by the FL (*TF3a*) and B-LBL (*TF3b*) (Supplementary Table 1). These genes were previously described to be mutated in FL.[5, 22-26] *KMT2D* has a role in epigenetic programming and is considered as a driver gene of FL.[22] As compared to the preceding FL (*TF3a*), seven missense mutations and one nonsense mutation were gained in the B-LBL stage. One of the missense mutations in *CCND3* (T283A) is located in exon 5, a region recurrently mutated in DLBCL and BL. In 38% of sporadic BL cases a mutation in *CCND3* is present.[47] The T283A mutation was shown to increase protein stability of *CCND3* conferring a proliferative advantage.[47] In the FL stage, a deletion of chr. 1p36 was detected, including deletion of *TNFRSF14*. In FL, -1p36 is found in frequencies between 20 – 40% and has been correlated with dismal prognosis.[28, 48] Interestingly, in addition to -1p36, in the *TF3b* B-LBL a nonsense mutation in *TNFRSF14* was found, suggestive for a tumor suppressor role in the progression of FL. However, there are conflicting reports as to whether the combination of -1p36 with *TNFRSF14* mutation is associated with good or bad overall survival.[49, 50] Interestingly, the mutation in *SMARCA2* (*BRM*), which is part of the SWI/SNF ATP-dependent chromatin remodeling complex, was hemizygously found in the B-LBL stage due to LOH on chromosome 9p.[51] Mutations in *SMARCA2* and *SMARCA4* were described in marginal zone B-cell lymphoma[51], BL and DLBCL.[47] SMARCA2 is a

known interaction partner of C/EBPα, which forms heterodimers with C/EPBβ.[52] It has been shown that C/EPBα and C/EBPβ are involved in B-cell reprogramming to macrophage-like cells or stem cell-like cells. However, it is unclear whether SMARCA2 interacts with the heterodimer of C/EPBα and β and whether this interaction is necessary for de role in B-cell reprogramming.[53] In addition, SWI/SNF components, including SMARCA2, are necessary for the accessibility of the *CD79A* promotor for the transcription factors PAX5 and EBF1.[54] Via these mechanisms the identified mutation in *SMARCA2* may contribute to the lymphoma de-differentiation. Some of the mutations that were specifically and uniformly detected in the B-LBL (in *CCND3*, *ISOC2*, *MYC* and *ZFHX4)* were detectable in 3-9% of the sequence reads in the preceding FL timepoint. This observation indicates that minor precursor clones containing these mutations were present in the FL. One subclone of the FL with all these mutations eventually grew out as the B-LBL.

In a recently published study by Geyer et al. seven FLs, which transformed into B-LBLs, were analyzed. Similar to our study, all transformation cases were characterized by loss of follicular architecture, gain of TdT expression and the presence of a *MYC* gene rearrangement. The lymphoblastic lymphoma of one case was analyzed by whole-exome sequencing also identifying mutations in *TNFRSF14* and *CCND3*. Unfortunately, it is unknown whether these mutations had been present in the FL, as the preceding FL was not analyzed.[12]

Some of the genomic mutations acquired in the B-LBL are also implicated in the transformation from FL to DLBCL, such as deletion of *CDKN2A/B* and *MYC* translocation. *MYC* translocation is also essential in BL development and the acquired *CCND3* mutation (T283A) is frequently mutated in this aggressive entity. Although we also determined several acquired mutations, which were not described in either DLBCL or BL and intriguingly all FLs transforming to B-LBL expressed IgG, we cannot explain this peculiar transformation.

It was previously hypothesized that clonally related FLs and B-LBLs do not develop sequentially but might evolve in parallel from precursor cells harboring the t(14;18).[8, 37] A $t(14;18)^+$ sIG^- and TdT^+ B-cell might thus evolve into a TdT^-CD20^+ FL or

415 expand as a precursor B cell by acquisition of an additional *MYC* translocation. However,
416 more recent work has shown that after the acquisition of the t(14;18) in bone marrow
417 precursor B cells, these t(14;18) positive B cells further mature to memory B cells and
418 acquire somatic hypermutations in successive rounds of re-entry in germinal centres.[55]
419 Thus the B-LBL might originate from mature post-germinal center B cells functioning as
420 FL precursor cells that undergo a further round of re-entry in a germinal center, thereby
421 acquiring an AID induced MYC breakpoint. For still unknown reasons they also show
422 partial regression to a phenotype of precursor B cells. Our study, however, demonstrates
423 that the B-LBLs evolve from FL subclones that already accumulated the vast majority
424 (for *TF2* all) of the somatic *IGHV* mutations and for *TF3* also the majority of the genomic
425 aberrations. Whatever the exact mechanism may be, these data clearly show that the FL
426 and B-LBL pairs did not arise in parallel from t(14;18)$^{+}$ IG unmutated precursor cells.
427

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Authorship contributions

L.M.S., R.H., L.A.S., R.J.B. and C.J.M.vN. designed the research; L.M.S., R.H., L.A.S., T.A.M.W, M.E.C.M.O, E.J.M.S-T., A.B.M. and R.J.B. performed the research; P.M.K. provided patient material; L.M.S., R.H., L.A.S., A.J., J.E.J.G., R.J.B. and C.J.M.vN analyzed the data; L.M.S., R.H., L.A.S., R.J.B. and C.J.M.vN. wrote the manuscript; and all authors edited the manuscript.

Conflict of interest disclosure

The authors declare no competing financial interests.

Table 1. Four patients with a FL that has transformed into a B-LBL

Patient	Sex	Age	Diagnosis	Years from FL diagnosis	Location
TF1a	F	57	FL	0	lymph node
TF1b			B-LBL	2	epicardium
TF2a	F	51	FL	0	lymph node
TF2b			B-LBL	1	mouth
TF2c			B-LBL	2	mouth
TF3a	F	24	FL	0	lymph node
TF3b			FL	2	lymph node
			B-LBL	2	lymph node
TF4a	M	54	FL	0	lymph node
TF4b			DLBCL	1	abdominal wall
TF4c			B-LBL	1	retroperitoneum

B-LBL, precursor B-lymphoblastic lymphoma; FL, follicular lymphoma ; F, female; M, male.
In *TF1b*, *TF2b and TF4b* the pre-existing FL was no longer present, whereas in *TF3b* both the FL and B-LBL were detected. The biopsies of *TF2b* and *TF2c* were both taken from the mucosa of the jaw.
Patients *TF1, TF2*, *TF3* and *TF4* all died within two years after FL diagnosis.

Table 2. Immunophenotypes of four lymphomas before and after progression.

Patient	Diagnosis	CD20	FDC	CD10	BCL6	BCL2	TdT	Pax5	CD79a	CD19	Igλ	Igκ
TF1a	**FL**	+	+	+	+	+	-	+	+	+	+	-
TF1b	**B-LBL**	-	**nd**	+	-	+	+	+	+	+	+	-
TF2a	**FL**	+	+	+	+	+	-	+	+	**nd**	**nd**	**nd**
TF2b	**B-LBL**	-	**nd**	+	+	+	+	+	+	+	+	-
TF2c	**B-LBL**	-	**nd**	+	+	+	-	+	+	+	+	-
TF3a	**FL**	+	+	+	+	+	-	+	+	**nd**	**nd**	**nd**
TF3b	**FL**	+	+	+	+	+	-	+	+	+	**nd**	**nd**
	B-LBL	-	-	+	-	+	+	+	+	+	**nd**	**nd**
TF4a	**FL**	+	**nd**	+	+	+	-	+	+	+	**nd**	**nd**
TF4b	**DLBCL**	-	-	+	-	+	-	+	+	**nd**	**nd**	**nd**
TF4c	**B-LBL**	-	-	+	-	+	+	+	+	**nd**	**nd**	**nd**

Protein expression of CD20, CD21-L, CD10, BCL6, BCL2, TdT, Pax5, CD79a, CD19 and Igλ, as determined by immunohistochemistry (IHC) on FFPE and frozen sections. B-LBL, precursor B-cell lymphoblastic lymphoma; FL, follicular lymphoma; LN, lymph node; FDC, follicular dendritic cells (as determined by CD21-L staining); nd, not determined due to lack of appropriate patient material.

Table 3. *IGHV* and *IGLV* rearrangements of the four FLs and in their ensuing B-LBL.

Patient	Diagnosis	Subclass	IGHV-rearrangment	Mut# (shared with FL)	IGLV-rearrangement
TF1a	FL	IgG	IGHV4-61/na/IGHJ5a	22	IGLV2-18/IGLJ2/3a
TF1b	B-LBL	IgG	IGHV4-61/na/IGHJ5a	27 (19)	IGLV3-21/IGLJ3b
TF2a	FL	IgG	IGHV3-20/na/IGHJ6b	23	IGLV1-36/IGLJ3b
TF2b	B-LBL	IgG	IGHV3-20/na/IGHJ6b	23 (23)	polyclonal
TF3a	FL	Nd	IGHV1-69/IGHD1-26/IGHJ6c	10*	nd
TF3b†	FL ($CD10^{hi}$)	IgG	IGHV1-69/IGHD1-26/IGHJ6c	17	IGLV4-69/IGLJ2/3a
	FL ($CD10^{low}$)	IgG	IGHV1-69/IGHD1-26/IGHJ6c	15	IGLV4-69/IGLJ2/3a
	B-LBL (S)	IgG	IGHV1-69/IGHD1-26/IGHJ6c	15 (14)	IGLV4-69/IGLJ2/3a
	B-LBL (L)	IgG	IGHV1-69/IGHD1-26/IGHJ6c	15 (14)	IGLV4-69/IGLJ2/3a
TF4a	FL	IgG	IGHV3-73/IGHD1-7/IGHJ5*02	21	nd
TF4b	DLBCL	nd	nd	nd	nd
TF4c	B-LBL	IgG	IGHV3-73/na/ IGHJ5*02	25 (18)	nd

* *IGHV* SHM was determined starting from framework (FR) 1 in all instances, except for *TF3a*, in which SHM were determined starting from FR2 and *TF4*, in which SHM could not be determined in the first part of FR1.
† Lymph node suspensions of *TF3b* were sorted in 4 fractions (See Figure S1 and text).
na, not applicable.
nd, not determined

Table 4. Chromosomal translocations detected by FISH analysis.

Patient	BCL2	MYC	BCL6
TF1a	+	-	+
TF1b	+	+	-
TF2a	ni	-	-
TF2c	+	+	+
TF3a	+	-	-
TF3b	+	+	-
TF4a	+	-	-
TF4b	+	-	nd
TF4c	+	+	-

ni: not interpretable; nd: not determined

Table 5. Numerical genomic aberrations in *TF3*.

Region	FL	B-LBL	Remarks
1p36	del.	del.	Associated with transformation of FL
2p16	CN-LOH	CN-LOH	Ampl. associated with transformation of FL
5p12	CN-LOH	CN-LOH	Ampl. associated with transformation of FL
6q21	CN-LOH	CN-LOH	Del. in 17% of FL[28]
6q22	CN-LOH	CN-LOH	Ampl. associated with transformation of FL
7q33	CN-LOH	CN-LOH	LOH in 11% of FL[28]
8q	ampl.	ampl.	Includes ampl. of *MYC*
9p	wt	CN-LOH	Includes del. of *CDKN2*
14q32	wt	ampl.	Includes ampl. of *TCL1* and *BCL11*
17q12	wt	del.	Includes del. of *NF-1*
22q11	del.	del.	Del. in 10% of FL[28]

del.: deletion; CN-LOH: copy neutral loss of heterozygosity; ampl.: amplification; wt: wildtype

Table 6. Exome mutations gained in the B-LBL of *TF3b*.

Gene	Chromosome	Position	Mutationtype	RefSeq	Mutation	FL	B-LBL
BCL2	18	60986079	5'UTR	NM_00633	5UTR-180 C/T	wt	het
CCND3	6	41903710	missense	NM_001760	K268R (A803G)	wt	het
		41903754	missense		T283A (A847G)	wt	het
DYSF	2	71908237	missense	NM_01130987	P2057Q (C6170A)	wt	het
ISOC2	19	55967760	missense	NM_01136201	G94A (A32T)	wt	het
MYC	8	128748820	5'UTR	NM_002467	5UTR+19 T/C	wt	het
SMARCA2	9	2081868	missense	NM_003070	N741H (A2221C)	het	hem
SNAPC3	9	15451376	missense	NM_001039697	T264S (A791C)	het	hem
TNFRSF14	1	2488138	nonsense	NM_003820	W12* (G35A)	wt	hom
ZFHX4	8	77763412	missense	NM_024721	H1419Y (C4255T)	wt	het

wt: wildtype; het: heterozygous; hom: homozygous; hem: hemizygous

Figure legends

Figure 1: Histology and immunophenotype of *TF3* before and after transformation.
TF3a showed a follicular growth pattern and expressed CD20, BCL6 and BCL2. After transformation part of the tumor cells of *TF3b* retained a follicular growth pattern (as indicated by an arrowhead), which was alternated by sheets of lymphoblastic cells (as indicated by an arrow). The FL cells still expressed CD20, BCL6 and BCL2, whereas the B-LBL cells lacked expression of CD20 and BCL6 but expressed TdT.

Figure 2: *IGHV* configurations of FL and B-LBL.
Schematic representation of *IGHV* consensus sequences of *TF1-4* before and after progression. Vertical bars correspond to shared mutations between the FL and B-LBL. V corresponds to two shared mutations in the same codon. Non-shared replacement and silent mutations are indicated by closed and open lollipop symbols, respectively. Codon numbering is according to V-Quest (http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=humanIg).[18] Black boxes indicate parts of the *IGHV* gene that were not sequenced. ICV denotes the degree of intraclonal variation.

Figure 3: VpreB and lambda5 are expressed in the B-LBL but not in the FL.
RT-PCR analysis of *VpreB* and *Lambda5* expression before and after progression. B2M: β-2-microglobulin. *TF3* was sorted in four fractions (see figure S1 and text). The – indicates a negative H_2O control and the pre-B-cell line Nalm-6 was used as a positive control (+).

Figure 4: Genomic aberrations of *TF3b* FL and B-LBL as determined by comparative genomic hybridization.
(A) Overview of genomic aberrations of the four sorted cell populations of *TF3b* and two control FLs, as determined by comparative genomic hybridization. Red indicates homozygous deletion. Orange indicates heterozygous deletion. Green indicates copy-

neutral loss of heterozygosity (CN-LOH). Blue indicates 2-fold amplification. Purple indicates >3-fold amplification. (B) Allele frequency of chromosome 9p in the B-LBL of *TF3b*. The green area indicates CN-LOH. Red indicates deletion.

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FIGURE 1

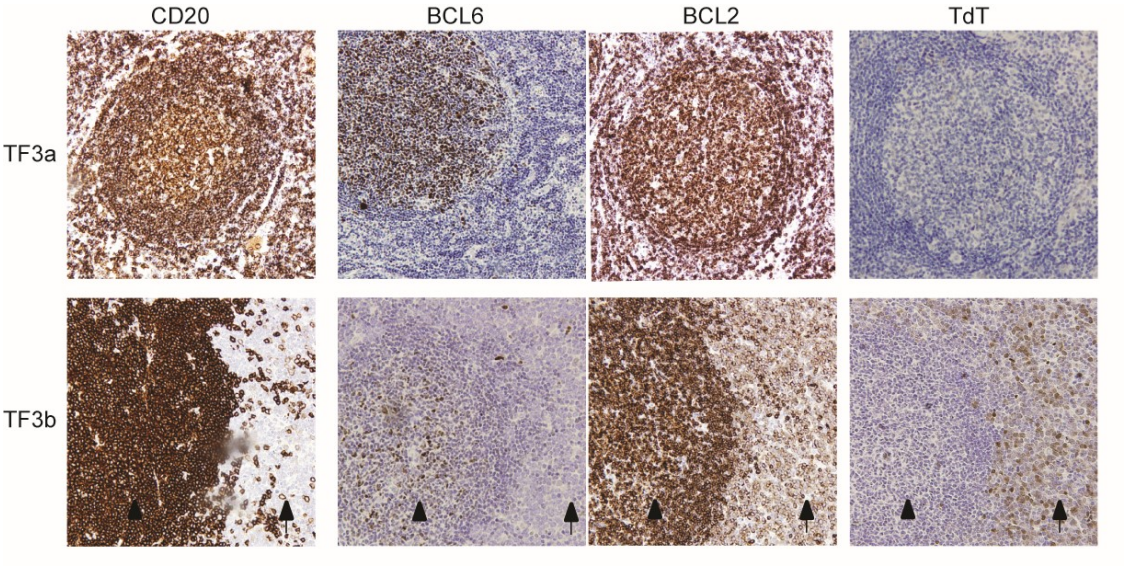


FIGURE 2

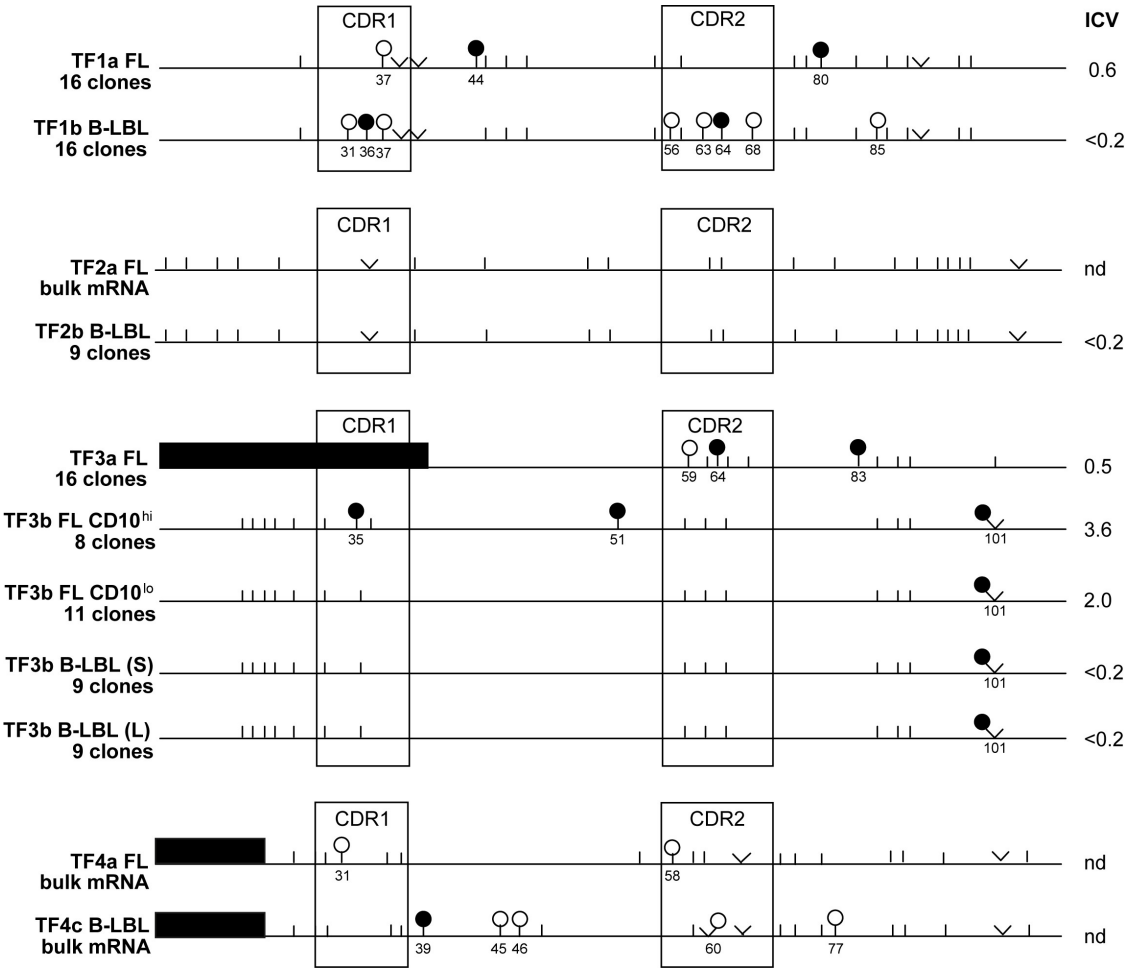


FIGURE 3

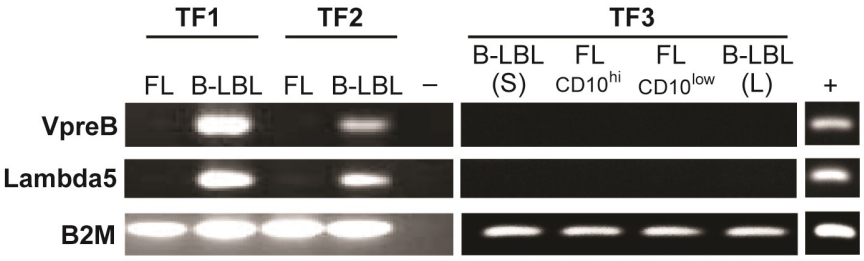
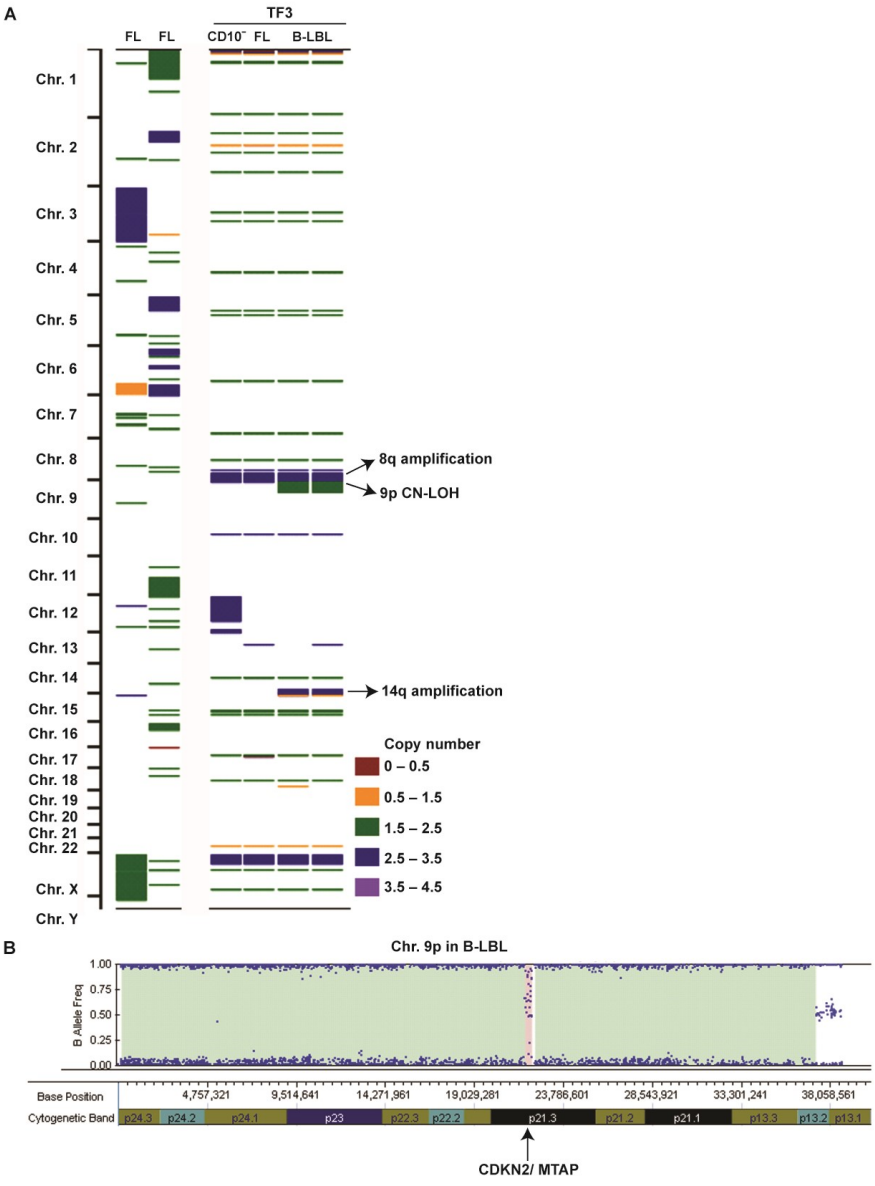


FIGURE 4


A
TF3
FL
FL
CD10⁻ FL
B-LBL
Chr. 1
Chr. 2
Chr. 3
Chr. 4
Chr. 5
Chr. 6
Chr. 7
Chr. 8
Chr. 9
Chr. 10
Chr. 11
Chr. 12
Chr. 13
Chr. 14
Chr. 15
Chr. 16
Chr. 17
Chr. 18
Chr. 19
Chr. 20
Chr. 21
Chr. 22
Chr. X
Chr. Y
8q amplification
9p CN-LOH
14q amplification
Copy number
0 – 0.5
0.5 – 1.5
1.5 – 2.5
2.5 – 3.5
3.5 – 4.5
B
Chr. 9p in B-LBL
B Allele Freq
1.00
0.75
0.50
0.25
0.00
Base Position
4,757,321
9,514,641
14,271,961
19,029,281
23,786,601
28,543,921
33,301,241
38,058,561
Cytogenetic Band
p24.3
p24.2
p24.1
p23
p22.3
p22.2
p21.3
p21.2
p21.1
p13.3
p13.2
p13.1
CDKN2/ MTAP